

RESEARCH

Open Access



Differences in amino acid frequency in CagA and VacA sequences of *Helicobacter pylori* distinguish gastric cancer from gastric MALT lymphoma

Masahiko Hashinaga^{1,2}, Rumiko Suzuki¹, Junko Akada¹, Takashi Matsumoto¹, Yasutoshi Kido¹, Tadayoshi Okimoto², Masaaki Kodama^{2,3}, Kazunari Murakami² and Yoshio Yamaoka^{1,4*}

Abstract

Background: *Helicobacter pylori* is a pathogenic bacterium that causes various gastrointestinal diseases. The most common gastric malignancies associated with *H. pylori* are gastric cancer and lymphoma of mucosa associated lymphoid tissue (MALT). *Helicobacter pylori* virulence genes, namely *cagA* and *vacA*, are known to be associated with malignancy development. Conventionally, *cagA* and *vacA* were classified by looking at partial sequences of the genes. However, such genotyping has hardly proven useful predicting different risks for gastric cancer or MALT lymphoma. In search of new loci that distinguish these diseases, we investigated the full sequences of *cagA* and *vacA*.

Results: We compared *cagA* and *vacA* sequences of 18 and 12 *H. pylori* strains obtained, respectively, from patients with gastric cancer and MALT lymphoma in Oita, Japan. Conventional genotyping of *cagA* and *vacA* showed no significant difference between the two diseases. We further investigated the full protein sequences of CagA and VacA to identify loci where allele frequency was significantly different between the diseases. We found four such loci on CagA, and three such loci on VacA. We also inspected the corresponding loci on the genes of 22 gastritis strains that potentially lead to gastric cancer or MALT lymphoma in the long run. Significant differences were observed at one CagA locus between gastritis and MALT lymphoma strains, and at one VacA locus between gastritis and gastric cancer strains.

Conclusions: We found novel candidate loci in *H. pylori* virulence genes in association with two different types of gastric malignancies that could not be differentiated by conventional genotyping. Biological connotations of the amino acid polymorphisms merit further study.

Keywords: *Helicobacter pylori*, *cagA*, *vacA*, Amino acid polymorphism, Next generation sequencing

Background

More than half of the world's population is infected with *Helicobacter pylori*, a gram-negative spiral bacterium whose ecological niche is the human stomach. The infection is associated with severe gastritis-associated diseases, including peptic ulcer disease, gastric cancer (GC),

and lymphoma of mucosa associated lymphoid tissue (MALT lymphoma) [1]. Most *H. pylori*-infected individuals develop histological gastritis, and about 10% of those infected develop severe diseases [2].

Epidemiological studies suggested that *H. pylori* infection plays an oncogenic role in both GC and MALT lymphoma [3–5]. Over their entire lifetimes, ~1 to 2% of *H. pylori* infected individuals develop GC, and less than 0.1% of them develop MALT lymphoma [6, 7]. The mechanisms for *H. pylori* involvement in these two kinds of malignant diseases remain unknown.

*Correspondence: yyamaoka@oita-u.ac.jp

¹ Department of Environmental and Preventive Medicine, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama-machi, Yufu, Oita 879-5593, Japan

Full list of author information is available at the end of the article

Helicobacter pylori is a highly heterogeneous species, and its virulence varies geographically [8]. The bacterial factors cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) are the most extensively studied *H. pylori* virulence factors. The major *H. pylori* virulence factor CagA is translocated into gastric cells via type IV secretion system and plays an important role in gastric carcinogenesis [9–11]. Injection of CagA requires a host cell receptor that was identified as integrin beta 1 [12, 13]. Recently there are several evidences that CagA also injected into B-cells in gastric MALT lymphoma [14, 15]. There are two types of clinical isolates: CagA-producing (*cagA*-positive) and CagA non-producing (*cagA*-negative) strains. The CagA protein contains a C-terminal region containing Glu-Pro-Ile-Tyr-Ala (EPIYA) tyrosine-phosphorylation motifs. The sequences are annotated according to segments (20–50 amino acids) flanking the EPIYA motifs (i.e., segments EPIYA-A, -B, -C, or -D) [16–18]. *cagA* containing EPIYA-D segments is typically observed in strains isolated from East Asia, and is denoted as East-Asian-type *cagA*; whereas *cagA* containing EPIYA-C segments is observed globally in non-East Asia strains, including those from Europe, South Asia, Africa, Australia, and the American continents, and is denoted Western-type *cagA* [19]. East-Asian-type *cagA* exhibits a stronger binding affinity for src homology-2 domain-containing phosphatase 2 (SHP2), and a greater ability to induce morphological changes in epithelial cells, than Western-type *cagA* [8, 16]. Our in vivo epidemiological studies also revealed a higher prevalence of East-Asian-type *cagA* strains in patients with GC than in patients with gastritis, and confirmed that East-Asian-type *cagA* is a significant risk factor for GC in Okinawa (Japan) and Thailand [20, 21]. Such different types of *H. pylori* virulence factor, at least in part, affect the geographic differences in incidence of gastric malignancies.

VacA is another extensively studied *H. pylori* virulence factor. Although nearly all strains possess the *vacA* gene. VacA protein possesses the capacity to induce cell vacuolization was found to differ significantly from strain to strain [22]. Four high sequence diversity regions of *vacA* have been found to closely associate with *H. pylori* vacuolating activity, including signal (s)-, intermediate (i)-, middle (m)-, and deletion (d)-regions. s, i, m, d regions are classified either type 1 or 2, respectively, and *vacA* s1m1 type is related with the higher risk of gastric malignancies [23, 24].

In Japan, almost all strains have ABD-type *cagA* and s1m1-type *vacA*, which are the most virulent genotypes, although clinical outcomes of the infection vary [25–27]. Conventional genotyping based on the partial sequences of the genes are not enough to predict different risks for gastric cancer or MALT lymphoma. Although the genetic

diversity of these factors affects the pathogenesis of associated malignancies, few previous studies have focused on the entire sequences of *cagA* and *vacA* [28]. We therefore investigated the full protein sequences of CagA and VacA to identify amino acid loci where the allele frequency is significantly different between *H. pylori* strains isolated from patients with GC and MALT lymphoma. The differing amino acid residues were further compared with their corresponding amino acids in *H. pylori* strains isolated from patients with gastritis, who principally were individuals who may develop either GC or MALT lymphoma in the future.

Methods

Patients and *H. pylori*

Helicobacter pylori strains were isolated from the gastric mucosa of Japanese patients infected with *H. pylori* who underwent endoscopy at Oita University Faculty of Medicine Hospital (Yufu, Japan) and its affiliated hospitals (Oita, Japan) between February 1997 and April 2014. Included presentations were gastritis only (i.e., histological gastritis without peptic ulcers nor any malignancy), GC, and MALT lymphoma. We analyzed 18, 12, and 22 strains obtained from patients with GC, MALT, and gastritis, respectively. For patients with GC, MALT, and gastritis, respectively, the average age and the (male/female ratio) were 64.3 a (9/9), 64.4 a (8/4), and 57.9 a (9/13). Gastric biopsy specimens were taken from the antrum (pyloric gland area) and the corpus (fundic gland area). GC were identified by endoscopy; GC and MALT lymphoma were further confirmed by histopathology [29]. Written informed consent was obtained from all participants, and the protocol was approved by the Ethics Committees of Oita University Faculty of Medicine (Yufu, Japan).

Isolation of *H. pylori*

Antral biopsy specimens were obtained for the isolation of *H. pylori* using standard culture methods, as previously described [25]. *Helicobacter pylori* DNA was extracted from confluent plate cultures expanded from a single colony using DNeasy Blood & Tissue kit (QIAGEN Inc., Valencia, CA, USA).

Gene sequencing and data acquisition

Genome sequence data of *H. pylori* strains were obtained using paired-end reads (2 × 150 or 2 × 300 bp) on HiSeq 2000 and MiSeq sequencers (Illumina, Inc., San Diego, CA, USA). Sample multiplexing was provided by using an Indexed DNA library, and manipulated using the Nextera XT Index Kit and the Nextera XT DNA Library Preparation Kit (Illumina, Inc., San Diego, CA, USA), following the manufacturer's instructions. We

performed de-novo assembly of the short read data using the CLC Genomics Workbench v. 7.0.4 commercial software (CLC QIAGEN), with automated word size and bubble size, and a minimum contig size 200 bp. ORFs were predicted by the same software, which identified CagA and VacA using the genes of Japanese strain F32 (Accession Number NC_017366). We took top hit genes whose % identity and length coverage were at least 85 and 80%, respectively. In case *cagA* and *vacA* genes were not detected by next-generation sequencing, we used PCR and the Sanger method. Primers for PCR amplification and direct sequencing of the entire coding regions of *cagA* and *vacA* are shown in Table 1. The regions containing full-length *cagA* and *vacA* were amplified by PCR, and sequenced as previously described. We attached a list of accession numbers of the *cagA* and *vacA* sequences as Additional file 1.

Comparison of amino acid frequency

We first investigated the genotypes of *cagA* and *vacA* by conventional motif analysis. East-Asian ABD-type

Table 1 Primers used for DNA sequencing of *H. pylori cagA* and *vacA* in this study

Gene	Primer	Sequence (5'–3')	References
<i>cagA</i>	Luni 1	ACATTTTGGCTAAATAAACACTG	[39]
	R5280	GTTGCACGCATTTTCCCTTGATC	[39]
	L2(+)	AAGGAGAAACAATGACTAACGAAAC-TATTG	[40]
	L2(-)	TCCTTTAAGATTTTTGGAAACCAC-CTTTTG	[40]
	hp552-R2	CTCTTACTCACCTTGATCCTCG-CATA	In this study
	hp552-R1	GCCTGGATCGCTCAAACCTGGCATGC	In this study
	cagA-R2	GGGTGTTGATTTTAGACGGATC	In this study
	cagA-F9	TCGTTCAAGTTTTCCACCAAGTTGA	In this study
	cagA-F11	CAATCAAGAGGCTAGTAAGGAAG	In this study
	cagA-F6	CTCTCAAAGATTATGGGAAAAA	In this study
	cagA-F10	ACAATAACGTTCTATCTTCTGTGCT	In this study
	cagA-F8	AAAGATCCGTCTAAAATCAACACCC	In this study
	TF	ACCCTAGTCGGTAATG	[20]
TR	TATCAGAAGCTAAAAC	[20]	
OMF	AGCAAAAAGCGACCTTGAAA	[20]	
OMR	ATTCACGAGCTTGAGCCACT	[20]	
<i>vacA</i>	vacAcysS-F2	GAATTTCAATGAAGAAGACTTGT	In this study
	vacA-R4	GCGGTGTGTTTGTGTATTCCAT	In this study
	VA1-F	ATGGAAATACAACAACACAC	[22]
	VAG-R	GCGTCAAAAATAATTCCAAGGC	[25]
	VAG-F	CAATCTTGCCAATCAAGCGAG	[25]
	VA3-F	GGTCAAATGCGGTCATGG	[22]
	vacAfecE-R2	CTTATTGTTACTGGATGAGCCTA	In this study

cagA sequences (13 GC strains and 8 MALT strains), and s1m1-type *vacA* sequences (15 GC strains and 10 MALT strains) were deposited to GeneBank (Accession No. LC185348-LC185429) and used for further analysis. We aligned protein sequences of the genes using MAFFT v6.717b. We next investigated difference of amino acid allele frequency between GC and MALT strains for each locus using an original Perl script (detailed information available upon request). The significance of each amino acid difference was tested using Fisher's exact test, as implemented by the R statistics software.

MLST analysis

To investigate whether the observed differences in amino acid frequency could be explained by population structure, we used multi locus sequence typing (MLST) methods to construct a phylogenetic tree. Seven housekeeping genes of MLST (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, and *yphC*) were obtained in the same manner as *vacA* and *cagA*. Specific portions of these genes were concatenated into sequences of 3406 bp each. We also downloaded from the PubMLST database 1439 reference strains with bacterial population information determined by STRUC-TURE analysis in previous studies (<http://pubmlst.org/helicobacter/>). We next integrated the reference sequences with our sequences, and constructed a phylogenetic tree using MEGA v. 6.0.

Results

Conventional genotyping of *cagA* and *vacA*

We first analyzed the genotypes of these genes using DNA sequences obtained by next generation sequencing (NGS) or Sanger methods. The *cagA* genotype was classified by its combination of C-terminal EPIYA region segments denoted as A, B, C, and D. *cagA* with ABC segments was classified as Western type; *cagA* with ABD segments was classified as East-Asian type (Table 2). We analyzed *cagA* sequences of 14 GC and 11 MALT strains obtained by either NGS or the Sanger method. Most GC strains (13/14 strains; 92.9%) possessed East-Asian type ABD *cagA*, except for one *cagA*-negative strain. While most MALT lymphoma strains (8/11; 72.7%) also possessed East-Asian type ABD *cagA*, we also identified 1 Western type ABCC *cagA*, 1 AB type, and 1 atypical East-Asian ABABD type. Recent data revealed a strong non-random distribution of the B-motifs (including EPIYA and EPIYT) in CagAs. The EPIYT B-tyrosine phosphorylation motifs (TPMs) were significantly less associated with GC than the EPIYA B-TPMs [30]. In this study, most strains possessed EPIYA B-TPMs, except for three strains with ESIYA or ESIYT B-TPMs (one strain from GC had ESIYA B-TPM, and one strain from gastritis and one strain from MALT lymphoma had ESIYT B-TPM).

Table 2 Strain used for *cagA* analysis

Gene	Type	Gastric cancer (n = 14)	MALT lymphoma (n = 11)
<i>cagA</i>	ABD	13	8
	ABABD	0	1
	AB	0	1
	ABC	0	0
	ABCC	0	1
	Negative	1	0
Entire <i>cagA</i> studied	ABD	13	8

Sequences of *vacA* were categorized by their combinations of s, m, i, and d regions (Table 3). We analyzed *vacA* sequences of 17 GC and 12 MALT strains obtained by either NGS or the Sanger method. Each region was classified into two types, such as s1 and s2; each type 1 signified virulence, and each type 2 signified non-virulence. Most GC strains (15/17 strains: 88.2%) were s1m1i1d1-type, while most MALT lymphoma strains (10/12: 83.3%) were also s1m1i1d1-type. Somewhat interestingly, d2 genotypes were observed only in MALT lymphoma strains (2/12 strains). However, as expected, most strains isolated from Japanese patients with GC and MALT lymphoma were East-Asian-type *cagA* and s1m1i1d1 *vacA* genotypes; we could not find the genotypes specific for those diseases in Japan. We therefore investigated entire sequences of *cagA* and *vacA* genes to identify disease-specific loci at which significant differences in amino acid frequency were observed between GC and MALT lymphoma strains. Since different genotypes possessed different motifs and repeats, we focused on *cagA* with ABD segments and *vacA* of s1m1i1d1 type, which possessed stronger virulence than other genotypes, and predominate among East Asian *H. pylori*.

Significant difference of amino acid frequency between samples of GC and MALT lymphoma

We detected disease-specific differences at four *cagA* loci in strain 26,695 (sites 314, 594, 684, and 1077) (left half of columns in Table 4; Fig. 1). At *cagA* site 314, asparagine

Table 3 Strain used for *vacA* analysis

Gene	Type	Gastric cancer (n = 17)	MALT lymphoma (n = 12)
<i>vacA</i>	s1m1i1d1	15	10
	s1m2i1d1	2	0
	s1m2i1d2	0	1
	s1m2i2d2	0	1
	Entire <i>vacA</i> studied	s1m1i1d1	15

(N) was dominant in the GC group, while serine (S) was the majority state in the MALT group. At site 594 of *cagA*, serine (S) was dominant in the GC group, while leucine (L) was the majority state in the MALT group. At site 684 of *cagA*, isoleucine (I) was dominant in the GC group, whereas the MALT group exhibited four amino acid variants, with valine (V) the most common among them. Among six kinds of amino acid substitution at *cagA* site 1077, serine (S) was dominant in the GC group; residues other than asparagine (N) exhibited similar frequencies in the MALT group. All four of these *CagA* loci differed significantly ($P < 0.05$, Fisher's exact test) between the 13 GC samples and the 8 MALT lymphoma samples.

We detected significant differences in three *vacA* loci at sites 672, 921, and 1037 in strain 26,695 (left half of Table 5; Fig. 2). At *vacA* site 672, isoleucine (I) was dominant in the GC group, while valine (V) was dominant in the MALT group. At *vacA* site 921, asparagine (N) predominated in both the GC and the MALT groups; however, some aspartic acid (D) substitutions occurred in the MALT group. At position 1037 of *vacA*, serine (S) predominated in both groups, but the frequencies of asparagine (N) and glycine (G) differed. All three of these *VacA* loci differed significantly ($P < 0.05$, Fisher's exact test) between the 15 GC samples and the 9 MALT lymphoma strains.

Absence of population structure confirmed by MLST phylogeny

If the samples were taken from different populations and disease prevalence was different between the populations, amino acid frequency could differ not because of the diseases but because of the populations. To test for structure within the studied strain population, we constructed phylogenetic trees based on MLST sequences. MLST uses seven housekeeping genes; therefore, a phylogenetic tree based on MLST reflects lineage differences that are independent of virulence. If adenocarcinoma strains and lymphoma strains were divided into two clusters in the MLST tree, amino acid differences may be irrelevant to disease type.

In the MLST phylogenetic tree with global strains, both GC and MALT lymphoma groups were located around East Asian strains (Additional file 2: Figure S1). The phylogenetic tree of only Japanese strains exhibited a radial branching shape, and GC and MALT lymphoma strains scattered randomly (Additional file 3: Figure S2). These trees imply an absence of population structure among the GC and MALT lymphoma samples.

Significant differences in loci between GC or MALT lymphoma and gastritis strains

The significant disease-specific *cagA* and *vacA* variations were not due to sample population differences. We

Table 4 Difference of amino acid frequency at loci: comparison of each disease in genotypes of *cagA* ABD type

Position	Amino acid residue		Gastric cancer (GC) (n = 13)	MALT (n = 8)	P value GC vs MALT	Gastritis (G) (n = 18)	P value	
	26,695	F32					G vs GC	G vs MALT
314G	310N	G	1	0	0.017	0	0.71	0.0078 (P < 0.01)
		N	11	2		16		
		S	1	4		1		
		Sequence gap	0	2		1		
594L	590S	L	3	6	0.032	5	1	0.081
		S	10	2		12		
		Sequence gap	0	0		1		
		Sequence gap	0	0		1		
684I	680V	I	11	2	0.011	9	0.33	0.63
		N	0	1		2		
		T	0	1		1		
		V	2	4		5		
		Sequence gap	0	0		1		
1077N	1064S	D	1	1	0.029	3	0.58	0.46
		H	1	2		2		
		N	4	0		4		
		S	7	2		5		
		T	0	3		2		
		G	0	0		2		

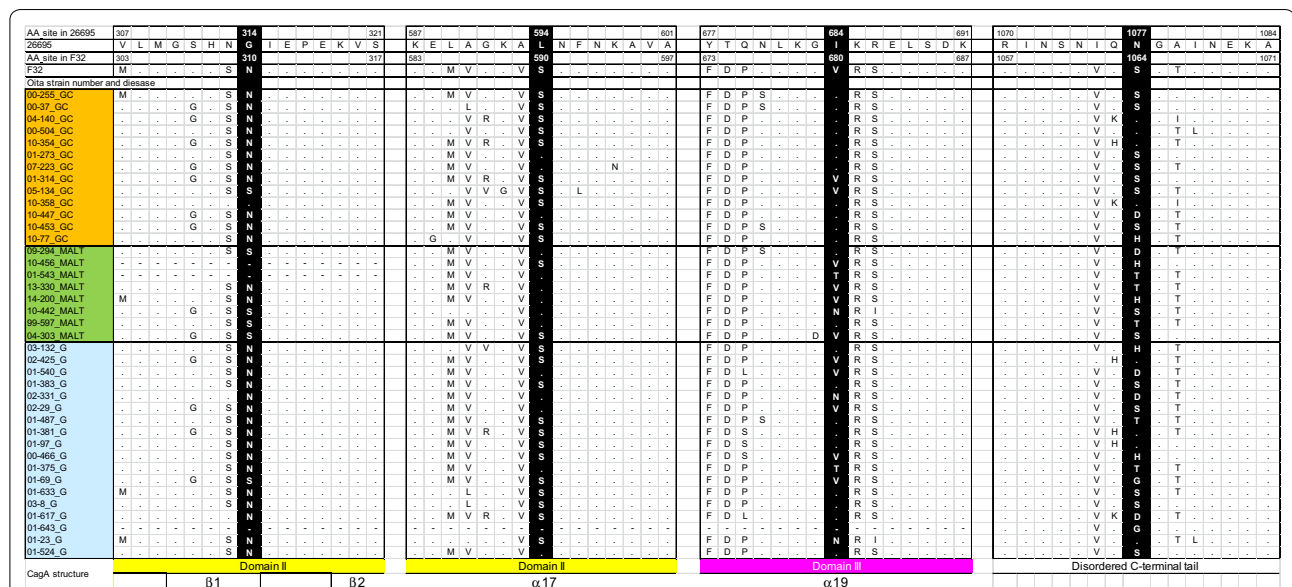


Fig. 1 CagA amino acid comparison between MALT lymphoma, gastric cancer, and gastritis strains. CagA amino acid comparison of strains 26,695, F32, and clinical isolates obtained in Oita, Japan are presented. Four significantly different loci are shown in white letters with black background. CagA structure was referred to [31]. GC gastric cancer, G gastritis

therefore compared loci in gastritis samples with their counterparts in GC and MALT lymphoma samples; these samples were obtained at the same hospitals in Japan. Both of the malignant diseases were presumed to be derived from gastritis, so we examined the same *cagA* and *vacA* loci for 22 gastritis strains, and compared these

observations with corresponding amino acid frequencies for GC and MALT lymphoma samples. Four *cagA* loci in gastritis samples (positions 314, 594, 684, and 1077) were checked (right half of Table 4; Fig. 1). We analyzed *cagA* sequences of 18 gastritis strains obtained by either NGS or the Sanger method. Two *cagA*

Table 5 Difference of amino acid frequency at loci: comparison of each disease in genotypes of *vacA* s1m1 type

Position		Amino acid residue	Gastric cancer (GC) (n = 15)	MALT (n = 10)	P value GC vs MALT	Gastritis (G) (n = 18)	P value	
26,695	F32						G vs GC	G vs MALT
672I	672I	I	9	1	0.033	8	0.49	0.19
		V	6	8				
		Sequence gap	0	1				
921D	921N	D	0	3	0.042	8	0.0036 (P < 0.01)	0.69
		N	15	6				
		Sequence gap	0	1				
1037N	1037S	S	13	6	0.042	16	0.16	0.30
		N	2	0				
		G	0	3				
		Sequence gap	0	1				

positions (684 and 1077) in the gastritis samples were not significantly different from their counterparts in GC or MALT lymphoma samples. Amino acid frequency of gastritis samples seems to be intermediate between GC and MALT lymphoma samples. However, *cagA* position 314 in gastritis samples was significantly different ($P = 0.008$, Fisher's exact test) from its counterpart in MALT lymphoma samples, but very similar to GC samples ($P = 0.71$). Also, *cagA* 594 in gastritis samples had the same tendency of near significant difference from MALT lymphoma samples ($P = 0.081$), but was indistinguishable from GC samples ($P = 1.0$). From this analysis, *H. pylori cagA* in patients with MALT lymphoma had the residues 314S and 594L, whereas the patients with gastritis and GC patient often had the residues 314N and 594S.

We next checked three *vacA* loci (positions 672, 921, and 1037) in gastritis samples (right half of Table 5; Fig. 2). Since we could not obtain *vacA* sequences from four gastritis strains (these strains lacked *cagA*), 18 sequences were analyzed. Two *vacA* positions (672 and 1037) of gastritis samples were not significantly different from their GC or MALT lymphoma counterparts. However, one *vacA* position (921) differed significantly between gastritis samples and GC samples ($P = 0.004$, Fisher's exact test). This position did not differ significantly between gastritis samples and MALT lymphoma samples ($P = 0.69$). The *H. pylori vacA* from patients with gastritis and MALT lymphoma assumed either a D or N state at position 921; the homologous position in GC patient *H. pylori* was a conserved N.

Discussion

We found disease-specific amino acid variations at four loci in ABD-type CagA (positions 314, 594, 684, and 1077 in strain 26,695), and at three loci in s1m1-type VacA (positions 672, 921, and 1037), some of which deviated significantly between GC strains and MALT lymphoma

strains. There were no differences in the amino acid frequency in all four CagA loci between GC and gastritis. Since gastritis cases in this study comprised atrophic gastritis—the precancerous phase—these results were not so surprising. However, CagA position 314 was significantly different between MALT lymphoma and gastritis strains. Most GC and gastritis strains contained asparagine (N) at position 314, but MALT lymphoma strains contained mainly serine (S) (4/6 sequenced strains). This difference suggested that N to S substitution at site 314 might be involved with the development of MALT lymphoma.

Recent studies have suggested that CagA would play a pivotal role in gastric MALT lymphoma pathogenesis [9, 14, 15]. CagA was indicated to translocate into human B-lymphocytes, hindering their apoptosis. This CagA translocation presumably leads to continuous B-lymphoid cell proliferation. Genetic variations of CagA in the development of MALT lymphoma are still unknown, although diversity has been indicated to affect the extent to which CagA causes inflammation to gastric epithelial cells. Here, we report disease-specific CagA sites that may discriminate between GC and MALT lymphoma.

From the view point of the CagA tertiary structure, deduced from the protein structure of the 26,695 reference strain was reported [31], and residue 314 is located in the middle region of CagA, in Domain II of β -sheet 1, continuing from N-terminal Domain I; position 594 is located in α -helix 17 of Domain II (Figs. 1, 3). Both loci are close to the basic amino acid cluster at α -helix 18 in Domain II where mediates CagA membrane association, so near plasma membrane of host cells. We also used structural homology modeling of MALT lymphoma strain (09-294) CagA (Fig. 4) to place the 314S residue in a solvent-exposed region of CagA. A disease-specific polymorphism at this site may easily interact with other downstream signal molecules just under the plasma membrane in B-lymphocytes. Tyrosine phosphorylation

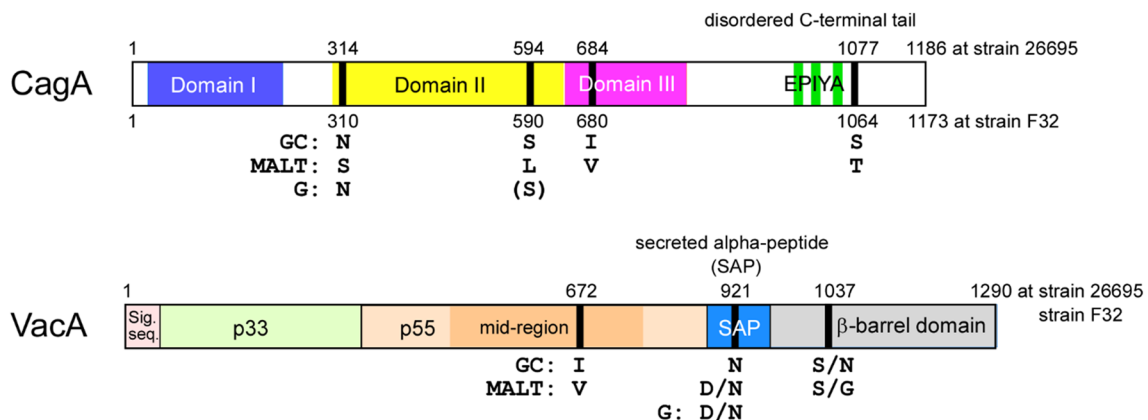


Fig. 3 Structures of CagA and VacA. Amino acid variations at four loci in ABD-type CagA (positions 314, 594, 684, and 1077 in strain 26,695) and those at three loci in s1m1-type VacA (positions 672, 921, and 1037); significant differences between gastric cancer strains and MALT lymphoma strains are presented

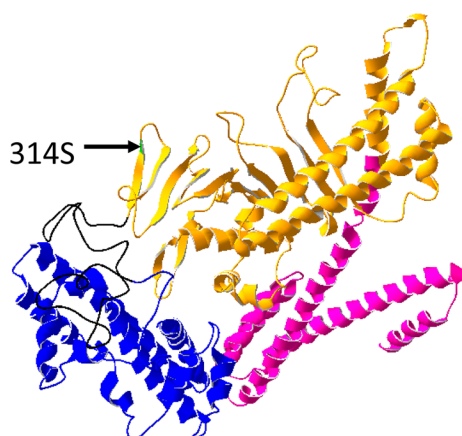


Fig. 4 Structural homology modeling of CagA. Three-dimensional structure of CagA derived from 09-294 (MALT lymphoma) was deduced by SWISS-MODEL [37, 38]. This model was constructed from the amino acid sequence of 09-294 from (residues 26F–819S), using 4dvy.1.A as a template. Domain I (26F–219F) is drawn in blue, Domain II (310G–638E) in yellow, and Domain III (644K–819S) in magenta. The arrow indicates the significant amino acid substitution 314S in MALT lymphoma, which is located in Domain II

between different diseases, some of the resulting loci may be polymorphisms important to the development of specific diseases. Further studies using isogenic mutants of the loci may be necessary to clarify the function of these amino acid differences. Second, many gastritis cases contained gastric atrophy since most infected Japanese had gastric atrophy in some degree. Therefore, most loci seemed to show no difference in amino acid frequency between GC and MALT lymphoma sequences. Although NGS technology is proposed wide variety of appreciation for biological problems [36], there are also some

limitations to using currently available NGS data. In most cases, NGS data alone could not obtain even the full *cagA* and *vacA* sequences; classical Sanger methods were necessary to confirm these sequences. The accuracy of NGS data is improving; in the near future we will use NGS to search for novel polymorphisms related to specific gastroduodenal diseases.

Despite these limitations, our methodology does offer a promising means of finding novel *H. pylori*-polymorphisms associated with specific diseases that involves NGS analysis.

Additional files

Additional file 1: Table S1. Accession numbers of the *cagA* and *vacA* gene sequences.

Additional file 2: Figure S1. Phylogenetic tree of the 18 GC and 12 MALT strains with 1439 PubMLST strains. This tree shows 18 GC and 12 MALT strains in 1439 global strains obtained from the PubMLST database. Orange and green triangles represent GC and MALT strains, respectively. hspEAsia, hspMaori, and hspAmerind are subpopulations of hpEastAsia. All GC and MALT strains were included within hpEastAsia population. (NJ-tree, Kimura-2 parameters, MEGA v. 6.0.).

Additional file 3: Figure S2. Phylogenetic tree of only 18 GC and 12 MALT strains. The star-like topology of this tree implies that these strains are genetically homogenous, and that their population has no clear structure (NJ-tree; Kimura-2 parameters; MEGA v. 6.0.).

Abbreviations

MALT: mucosa associated lymphoid tissue; GC: gastric cancer; CagA: cytotoxin-associated gene A; SHP2: src homology-2 domain-containing phosphatase 2; MLST: multi locus sequence typing; NGS: next generation sequencing.

Authors' contributions

YY and RS designed the study; MK, TO and, KM contributed to data collection; MH, RS, JA, TM, YK, and YY performed data analysis, performed data interpretation, and wrote the manuscript. MH, JA, TM and, RS aligned and analyzed sequences. YY revised the manuscript to include important content. All authors read and approved the final version of the manuscript.

Author details

¹ Department of Environmental and Preventive Medicine, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama-machi, Yufu, Oita 879-5593, Japan. ² Department of Gastroenterology, Faculty of Medicine, Oita University, 1-1 Idaigaoka, Hasama-machi, Yufu, Oita 879-5593, Japan. ³ Faculty of Welfare and Health Science, Oita University, 700 Dannoharu, Oita, Oita 870-1192, Japan. ⁴ Department of Medicine-Gastroenterology, Baylor College of Medicine and Michael E. DeBakey Veterans Affairs Medical Center, Houston, TX, USA.

Acknowledgements

We thank Prof. Tetsuya Hayashi and Dr. Yoshitoshi Ogura for supporting the next generation sequencing via the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) Genome Support Project. We also thank Ms. Yoko Kudo and Ms. Miyuki Matsuda for their excellent technical assistance.

Competing interests

The authors declare that they have no competing interests.

Availability of data and material

The *cagA* and *vacA* sequences in this study were deposited to GeneBank (Accession No. LC185348-LC185429).

Ethics approval and consent to participate

Ethics approval for research was obtained from the Ethics Committee of Oita University Faculty of Medicine, Yufu, Japan (P-12-10). All patients were adults and signed written informed consent for participation in this study.

Funding

This work was supported in part by grants from the National Institutes of Health (DK62813) and the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (25293104, 26640114, 15H02657, and 221S0002) (YY). This work was also supported by the Japan Society for the Promotion of Science (JSPS) Institutional Program for Young Researcher Overseas Visits (YY), and the Strategic Funds for the Promotion of Science and Technology from the Japan Science and Technology Agency (JST) (YY).

Received: 4 October 2016 Accepted: 26 October 2016

Published online: 08 November 2016

References

- Suerbaum S, Michetti P. *Helicobacter pylori* infection. N Engl J Med. 2002;347(15):1175–86.
- Wroblewski LE, Peek RM Jr, Wilson KT. *Helicobacter pylori* and gastric cancer: factors that modulate disease risk. Clin Microbiol Rev. 2010;23(4):713–39.
- Ferreira AC, Isomoto H, Moriyama M, Fujioka T, Machado JC, Yamaoka Y. *Helicobacter* and gastric malignancies. Helicobacter. 2008;13(Suppl 1):28–34.
- Conteduca V, Sansonno D, Lauletta G, Russi S, Ingravallo G, Dammacco F. *H. pylori* infection and gastric cancer: state of the art (review). Int J Oncol. 2013;42(1):5–18.
- Farinha P, Gascoyne RD. *Helicobacter pylori* and MALT lymphoma. Gastroenterology. 2005;128(6):1579–605.
- Polk DB, Peek RM Jr. *Helicobacter pylori*: gastric cancer and beyond. Nat Rev Cancer. 2010;10(6):403–14.
- Wang F, Meng W, Wang B, Qiao L. *Helicobacter pylori*-induced gastric inflammation and gastric cancer. Cancer Lett. 2014;345(2):196–202.
- Yamaoka Y. Mechanisms of disease: *Helicobacter pylori* virulence factors. Nat Rev Gastroenterol Hepatol. 2010;7(11):629–41.
- Ohnishi N, Yuasa H, Tanaka S, Sawa H, Miura M, Matsui A, Higashi H, Musashi M, Iwabuchi K, Suzuki M, et al. Transgenic expression of *Helicobacter pylori* CagA induces gastrointestinal and hematopoietic neoplasms in mouse. Proc Natl Acad Sci USA. 2008;105(3):1003–8.
- Tegtmeier N, Wessler S, Backert S. Role of the *cag*-pathogenicity island encoded type IV secretion system in *Helicobacter pylori* pathogenesis. FEBS J. 2011;278(8):1190–202.
- Backert S, Tegtmeier N, Fischer W. Composition, structure and function of the *Helicobacter pylori* *cag* pathogenicity island encoded type IV secretion system. Future Microbiol. 2015;10(6):955–65.
- Conradi J, Tegtmeier N, Woźna M, Wissbrock M, Michalek C, Gagell C, Cover TL, Frank R, Sewald N, Backert S. An RGD helper sequence in CagL of *Helicobacter pylori* assists in interactions with integrins and injection of CagA. Front Cell Infect Microbiol. 2012;2:70.
- Barden S, Lange S, Tegtmeier N, Conradi J, Sewald N, Backert S, Niemann HH. A helical RGD motif promoting cell adhesion: crystal structures of the *Helicobacter pylori* type IV secretion system pilus protein CagL. Structure. 2013;21(11):1931–41.
- Lin WC, Tsai HF, Kuo SH, Wu MS, Lin CW, Hsu PI, Cheng AL, Hsu PN. Translocation of *Helicobacter pylori* CagA into Human B lymphocytes, the origin of mucosa-associated lymphoid tissue lymphoma. Cancer Res. 2010;70(14):5740–8.
- Kuo SH, Chen LT, Lin CW, Wu MS, Hsu PN, Tsai HJ, Chu CY, Tzeng YS, Wang HP, Yeh KH, et al. Detection of the *Helicobacter pylori* CagA protein in gastric mucosa-associated lymphoid tissue lymphoma cells: clinical and biological significance. Blood Cancer J. 2013;3:e125.
- Higashi H, Tsutsumi R, Fujita A, Yamazaki S, Asaka M, Azuma T, Hatakeyama M. Biological activity of the *Helicobacter pylori* virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. Proc Natl Acad Sci USA. 2002;99(22):14428–33.
- Mueller D, Tegtmeier N, Brandt S, Yamaoka Y, De Poire E, Sgouras D, Wessler S, Torres J, Smolka A, Backert S. c-Src and c-Abl kinases control hierarchic phosphorylation and function of the CagA effector protein in Western and East Asian *Helicobacter pylori* strains. J Clin Invest. 2012;122(4):1553–66.
- Tegtmeier N, Wittelsberger R, Hartig R, Wessler S, Martínez-Quiles N, Backert S. Serine phosphorylation of cortactin controls focal adhesion kinase activity and cell scattering induced by *Helicobacter pylori*. Cell Host Microbe. 2011;9(6):520–31.
- Lind J, Backert S, Pfeleiderer K, Berg DE, Yamaoka Y, Sticht H, Tegtmeier N. Systematic analysis of phosphotyrosine antibodies recognizing single phosphorylated EPIYA-motifs in CagA of Western-type *Helicobacter pylori* strains. PLoS ONE. 2014;9(5):e96488.
- Matsunari O, Shiota S, Suzuki R, Watada M, Kinjo N, Murakami K, Fujioka T, Kinjo F, Yamaoka Y. Association between *Helicobacter pylori* virulence factors and gastroduodenal diseases in Okinawa, Japan. J Clin Microbiol. 2012;50(3):876–83.
- Vilaichone RK, Mahachai V, Tumwasorn S, Wu JY, Graham DY, Yamaoka Y. Molecular epidemiology and outcome of *Helicobacter pylori* infection in Thailand: a cultural cross roads. Helicobacter. 2004;9(5):453–9.
- Atherton JC, Cao P, Peek RM Jr, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. J Biol Chem. 1995;270(30):17771–7.
- Thi Huyen Trang T, Thanh Binh T, Yamaoka Y. Relationship between *vacA* types and development of gastroduodenal diseases. Toxins. 2016;8(6):182.
- Foegeding NJ, Caston RR, McClain MS, Ohi MD, Cover TL. An overview of *Helicobacter pylori* VacA toxin biology. Toxins. 2016;8(6):173.
- Yamaoka Y, Kodama T, Kita M, Imanishi J, Kashima K, Graham DY. Relationship of *vacA* genotypes of *Helicobacter pylori* to *cagA* status, cytotoxin production, and clinical outcome. Helicobacter. 1998;3(4):241–53.
- Azuma T, Yamazaki S, Yamakawa A, Ohtani M, Muramatsu A, Suto H, Ito Y, Dojo M, Yamazaki Y, Kuriyama M, et al. Association between diversity in the Src homology 2 domain-containing tyrosine phosphatase binding site of *Helicobacter pylori* CagA protein and gastric atrophy and cancer. J Infect Dis. 2004;189(5):820–7.
- Lind J, Backert S, Hoffmann R, Eichler J, Yamaoka Y, Perez-Perez GI, Torres J, Sticht H, Tegtmeier N. Systematic analysis of phosphotyrosine antibodies recognizing single phosphorylated EPIYA-motifs in CagA of East Asian-type *Helicobacter pylori* strains. BMC Microbiol. 2016;16(1):201.
- Yamazaki S, Yamakawa A, Okuda T, Ohtani M, Suto H, Ito Y, Yamazaki Y, Keida Y, Higashi H, Hatakeyama M, et al. Distinct diversity of *vacA*, *cagA*, and *cagE* genes of *Helicobacter pylori* associated with peptic ulcer in Japan. J Clin Microbiol. 2005;43(8):3906–16.
- Rugge M, Correa P, Dixon MF, Hattori T, Leandro G, Lewin K, Riddell RH, Sipponen P, Watanabe H. Gastric dysplasia: the Padova international classification. Am J Surg Pathol. 2000;24(2):167–76.

30. Zhang XS, Tegtmeyer N, Traube L, Jindal S, Perez-Perez G, Sticht H, Backert S, Blaser MJ. A specific A/T polymorphism in Western tyrosine phosphorylation B-motifs regulates *Helicobacter pylori* CagA epithelial cell interactions. *PLoS Pathog*. 2015;11(2):e1004621.
31. Hayashi T, Senda M, Morohashi H, Higashi H, Horio M, Kashiba Y, Nagase L, Sasaya D, Shimizu T, Venugopalan N, et al. Tertiary structure-function analysis reveals the pathogenic signaling potentiation mechanism of *Helicobacter pylori* oncogenic effector CagA. *Cell Host Microbe*. 2012;12(1):20–33.
32. Gangwer KA, Mushrush DJ, Stauff DL, Spiller B, McClain MS, Cover TL, Lacy DB. Crystal structure of the *Helicobacter pylori* vacuolating toxin p55 domain. *Proc Natl Acad Sci USA*. 2007;104(41):16293–8.
33. Gangwer KA, Shaffer CL, Suerbaum S, Lacy DB, Cover TL, Bordenstein SR. Molecular evolution of the *Helicobacter pylori* vacuolating toxin gene vacA. *J Bacteriol*. 2010;192(23):6126–35.
34. Yahiro K, Hirayama T, Moss J, Noda M. New insights into VacA intoxication mediated through its cell surface receptors. *Toxins*. 2016;8(5):152.
35. Bumann D, Aksu S, Wendland M, Janek K, Zimny-Arndt U, Sabarth N, Meyer TF, Jungblut PR. Proteome analysis of secreted proteins of the gastric pathogen *Helicobacter pylori*. *Infect Immun*. 2002;70(7):3396–403.
36. Nowrousian M. Next-generation sequencing techniques for eukaryotic microorganisms: sequencing-based solutions to biological problems. *Eukaryot Cell*. 2010;9(9):1300–10.
37. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics*. 2006;22(2):195–201.
38. Biasini M, Bienert S, Waterhouse A, Arnold K, Studer G, Schmidt T, Kiefer F, Gallo Cassarino T, Bertoni M, Bordoli L, et al. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res*. 2014;42(Web Server issue):252–8.
39. Mukhopadhyay AK, Kersulyte D, Jeong JY, Datta S, Ito Y, Chowdhury A, Chowdhury S, Santra A, Bhattacharya SK, Azuma T, et al. Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta, India. *J Bacteriol*. 2000;182(11):3219–27.
40. Satomi S, Yamakawa A, Matsunaga S, Masaki R, Inagaki T, Okuda T, Suto H, Ito Y, Yamazaki Y, Kuriyama M, et al. Relationship between the diversity of the cagA gene of *Helicobacter pylori* and gastric cancer in Okinawa, Japan. *J Gastroenterol*. 2006;41(7):668–73.

Submit your next manuscript to BioMed Central
and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at
www.biomedcentral.com/submit

